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Forensic interlaboratory evaluation of the ForFLUID kit for vaginal fluids identification

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ABSTRACT

Identification of vaginal fluids is an important step in the process of sexual assaults confirmation. Advances in both microbiology and molecular biology defined technical approaches allowing the discrimination of body fluids. These protocols are based on the identification of specific bacterial communities by microfloraDNA (mfDNA) amplification. A multiplex real time-PCR assay (ForFLUID kit) has been developed for identifying biological fluids and for discrimination among vaginal, oral and fecal samples. In order to test its efficacy and reliability of the assay in the identification of vaginal fluids, an interlaboratory evaluation has been performed on homogeneous vaginal swabs. All the involved laboratories were able to correctly recognize all the vaginal swabs, and no false positives were identified when the assay was applied on non-vaginal samples. The assay represents an useful molecular tool that can be easily adopted by forensic geneticists involved in vaginal fluid identification.

1. Introduction

According to the Department of Justice there is an average of more than 200,000 victims (age 12 or older) of rape and sexual assault each year in the USA.[1] Whilst in the past during sexual intercourses crime perpetrators did not pay much attention to wear protection barrier devices to decrease the probability of diseases or to avoid leaving their biological material, in the last few years the use of condoms or even gloves have been reported in such situations. In these cases semen stains or other body fluids of the offender are difficult to find on victims, thus reducing the probability of identification of the sexual abuser. A successful attempt to find valuable evidence to link the abuser to the crime is then dependent on finding saliva, fecal or vaginal fluid traces of the victim on the perpetrator pertinences. A relevant support in locating such kind of stains on objects, clothes or furniture may come from the use of alternate forensic light sources, which allow the presumptive detection of traces but do not confirm their biological origin.[2] It is worth noting that, whilst DNA typing of stains is necessary for the

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identification of the donor, DNA alone is always not enough to contextualize the stain to the crime, especially in sexual abuse cases. Thus the individualization of the nature of the stain is utmost essential to gather as much contextual information from biological evidences as possible during both the investigations and the trial.[2]

Several protocols have been described for the identification of vaginal fluids, mainly based on histochemical reactions, mRNA profiling or miRNA profiling.[3–7] These approaches have varying degrees of non-specificity or they require procedures not routinely established in many forensic laboratories. Histological approaches based on Lugol's iodine reaction can generate false-positives while an obstacle of forensic RNA analysis is the instability of the molecule, mainly due to the ubiquitous abundance of ribonuclease (RNase) enzyme in the environment.[8–11] Recent studies have suggested that mRNA samples from crime scenes can be sufficiently stable for forensic use, but the low diffusion of standardized RNase free procedures in many forensic laboratories can strongly affect reliability of results,[12,13] thus limiting their effective use in operational forensic investigations.

An interesting alternative to the identification of body fluids by organic compounds and nucleic acids of human origin is the characterization of the bacteria present in several body fluids exposed to the external environment such as perspiration, saliva, feces or vaginal mucous. The human microbiome differs between regions of the body and for this reason the analysis of the microflora DNA (mfDNA) present in a forensic sample can be useful for determining its nature and origin.[14] In addition the bacterial concentration in human body fluids is in the range of 10^8–10^9 colony forming units/ml, a quantity that allows easy molecular analysis also on tiny stains on clothes or surfaces.[15,16] This high abundance of bacteria living on the human body has allowed scientists to identify human contact traces on computer keys and computer mice just by nucleic acid tests for skin-associated bacteria.[17]

Recently, a multiplex real-time PCR assay (ForFLUID kit), based on the identification of commensal bacteria genomes, has been developed as a response to the problems described above.[18] The amplification of prokaryotic genomic DNA can lead to elevated sensitivity also in old samples due to the high stability of the template. In addition, the adopted extraction protocol is fully compatible with STR profiling, an important step in the confirmation of the human origin of the sample. The multiplex real-time amplification of genomic DNA of six microbes belonging to different body areas is also another relevant feature of the assay, particularly important in the identification of mixed samples.[18] The ForFLUID kit is able to amplify the genomic DNA of *Lactobacillus gasseri*, *Lactobacillus crispatus*, *Streptococcus salivarius*, *Streptococcus mutans*, *Enterococcus faecalis*, *Staphylococcus aureus*. *Lactobacilli* characterize vaginal bacterial flora and they can be detected in different physiological (eg. menses) or pathological (eg. bacterial vaginosis, menopause) conditions.[16,18]

In order to evaluate efficacy and reliability of the ForFLUID kit in the forensic identification of vaginal fluid an interlaboratory exercise has been performed by a blind trial proficiency test approach. In particular the results were collected from eight forensic laboratories, from different countries and using different real-time PCR thermal cyclers. All the laboratories received five identical swabs from homogeneous sets and one package of ForFLUID kit from a single batch, while they were asked to self provide DNA extraction and real-time PCR reagents and instruments.

2. Subjects & methods

2.1. Samples

Several different vaginal samples, described below, were provided (shipped in dry-ice) by the coordinating laboratory, to eight forensic biology institutes from different countries, involved in the blind proficiency test.

Vaginal fluids from four adult healthy women were obtained from specula after gynecological examination. Each speculum was washed with 5 ml sterile Dulbecco's Phosphate Buffered Saline (Sigma–Aldrich, St. Louis, MO, USA). The vaginal fluids obtained were then spotted (approximately 80 μl) on sterile cotton swabs (Heinz Herenz, Hamburg, Germany). Each sample of vaginal fluid has been sufficient to prepare more than 10 swabs. At the end, four sets of swabs (labeled v1–4) with vaginal fluids were ready for interlaboratory evaluation. A last set of swabs (labeled v11), spotted only with sterile buffer, was prepared as blind negative control. Every laboratory involved in the test received one swab from each set, together with a ForFLUID kit (same batch for all participants), a protocol derived from Giampaoli et al.[18] and a Questionnaire where they had to describe their practical execution of the test and eventual modifications to the suggested protocol (DNA extraction protocol; Real-Time PCR conditions and Analysis settings; Results). In addition they were invited to examine a few additional samples, self provided, selected from real forensic casework material, clinical or environmental material that could include human saliva/vaginal fluids/fecal stains to evaluate the assay performance also in complex samples from operational contexts.

2.2. DNA extraction

Every laboratory involved in the evaluation was asked to perform DNA extraction using commercially available kits specifically dedicated to Gram-positive bacteria. The large majorities of laboratories adopted the protocol described in Giampaoli et al.[18] using the GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich). Few laboratories adopted other protocols, in particular the QIAamp DNA mini kit (protocol for isolation of genomic DNA from Gram-positive bacteria) and the Promega DNA IQ Casework Pro Kit for Maxwell 16 with initial treatment with lysozyme (10 mg/ml). All the laboratories were warned on the importance of a strong initial lysis step, to be performed enzymatically and/or mechanically. Final elution was in 30–60 μl of water or TE buffer (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0).

2.3. Real-time PCR conditions

Real-time PCR reactions were performed in a final volume of 25 μl and 40 cycles of amplification. In order to mitigate eventual PCR inhibitor every laboratory was asked to read each sample at two concentration: 1 μl and 11 μl of DNA for reaction. The majority of participants adopted the TaqMan Universal Master mix II, no UNG (Life Technologies Corporation), but also the Takara Bio Premix Ex Taq (Takara Bio Inc., Otsu, Japan) was applied. Reactions were performed on Applied Biosystems real-time PCR thermocyclers (AB 7000, 7500, 7900 HT Fast; Life Technologies Corporation, Carlsbad, CA, USA), but also on Rotor-Gene 6000 (Corbett Life Science, Concorde, NSW, Australia). The thermocycler settings were as follows: beginning denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

2.4. Results interpretation

Data analysis was performed according Giampaoli et al.[18] considering clear amplification signals for $C_T < 30$, weak signal for $30 < C_T < 35$, doubt signal for $C_T > 35$. For each sample both results from 1 to 11 μl of template reactions were analyzed: when data were not homogeneous, the reaction with stronger signal was considered as more accurate.

The identification of the biological origin of the sample is performed through the definition of a microbiological signature of six bacteria. For this reason, the presence of amplification signals for only *L. gasseri* and/or *L. crispatus* is not sufficient for interpretation as a vaginal fluid, but a comparative analysis must be performed between all bacteria. Table 1 represents a simplified interpretation procedure adopted for classification of a specific sample as vaginal.

3. Results

3.1. Vaginal swabs from homogeneous sets

All laboratories were able to perform DNA extraction and real-time amplification from swabs v1–4, and v11. Sample v11 (sterile buffer) did not show amplification signals at any concentration/condition, confirming the absence of background (see Table 2a and b).

Sample v1 gave clear positive signal (C_T between 25 and 29) for *L. gasseri* in 7 laboratories, while in one situation a weak signal ($C_T = 33$) was obtained. All the participants did not obtained amplification signals for the other five microbes, with the exception of two doubt signals for *E. faecalis* (C_T 36 and 37) but in reactions where *L. gasseri* was detected at C_T 23 and 26. In conclusion, seven groups clearly identified sample v1 as vaginal fluid, while one group obtained a result of highly probable identification. Accuracy (calculated only on clear identifications) and precision for sample v1 are of 88%.

Table 1
Simplified representation of the interpretation procedure adopted for classify a specific sample as vaginal.

L. gasseri and/or *L. crispatus*	Other bacteria	Interpretation
One or both signals with CT < 30	No signal	Identification as vaginal fluid (vf)[a]
One or both signals with CT < 30	One or more bacteria with CT > 35	Highly probable as vf[b]
One or both signals with CT < 30	One or more bacteria with 30 > CT > 35	Probable as vf
One or both signals with CT < 30	One or more bacteria with CT < 30	Can neither be identified nor excluded as vf
One or both signals with 30 > CT > 35	No signal	Highly probable as vf[b]
One or both signals with 30 > CT > 35	One or more bacteria with CT > 35	Highly probable as vf
One or both signals with 30 > CT > 35	One or more bacteria with 30 > CT > 35	Probable as vf
One or both signals with 30 > CT > 35	One or more bacteria with CT < 30	Can neither be identified nor excluded as vf
One or both signals with CT > 35	No signal	Probable as vf
One or both signals with CT > 35	One or more bacteria with CT > 35	Can neither be identified nor excluded as vf
One or both signals with CT > 35	One or more bacteria with 30 > CT > 35	Not very probable as vf
One or both signals with CT > 35	One or more bacteria with CT < 30	Not very probable as vf[c]
No signal	No signal	Can be excluded as vf[d]
No signal	One or more bacteria with CT > 35	Can be excluded as vf
No signal	One or more bacteria with 30 > CT > 35	Can be excluded as vf
No signal	One or more bacteria with CT < 30	Can be excluded as vf[e]

[a] Unequivocal attribution (identification) only if the human origin is confirmed (presence of human DNA).
[b] When present both signal for *L. gasseri* and *L. crispatus* the interpretation is really close to the unequivocal attribution (additional considerations are required).
[c] Specific considerations on bacterial species can change the interpretation to exclusion of vf.
[d] Only if procedural errors can be excluded or if the starting material was not limiting (verify presence of prokaryotic DNA).
[e] Probably it is possible to identify the sample as oral or fecal.

Seven laboratories found clear amplification signals for *L. gasseri* (C_T between 24 and 29) on sample v2, while one group obtained a weak result ($C_T = 34$). All the participants did not obtained amplification signals for the other five microbes, with the exception of one doubt signal for *E. faecalis* (C_T 39), but in combination with a detection of *L. gasseri* at C_T 25, and one weak signal for *E. faecalis* (C_T 34), in combination with a detection of *L. gasseri* at C_T 28.

All laboratories obtained a good amplification signal for *L. gasseri* in sample v3 (C_T between 21 and 27), but with the presence of doubt signal for other microbes: for this reason the majority of laboratories interpreted these results as highly probable vaginal fluid. The detailed analysis of single results, suggested a clear identification at least for one laboratory (see Table 2b). In one situation a weak signal for *E. faecalis* was reported (C_T 34), while generally only doubt signals were present for this species or for *S. aureus* ($C_T > 35$).

Sample v4 presented a double signal *L. gasseri* and *L. crispatus* in all tests with the exception of one laboratory that found a positive signal only for *L. crispatus*. Four laboratories found doubt signals ($C_T > 35$) also for *S. salivarius*, *S. mutans* and *E. faecalis*, but always in reactions where *Lactobacilli* were detected at C_T between 14 and 24.

3.2. Forensic samples self-provided by participants

Each laboratory involved in the evaluation was invited to test the protocol on self-provided samples. A total of 10 samples contaminated with saliva, 5 samples with vaginal fluids, and 2 samples with fecal material. All saliva samples were always negative for *L. gasseri* and *L. crispatus* or *E. faecalis* and *S. aureus*, but when tested for *S. salivarius* or *S. mutans* they showed at least one amplification signal. Two salivary samples were oral swabs collected from patients under antibiotic treatment (ampicillin) and in both cases *S. salivarius* was detected (weak signal C_T at 33–34). All vaginal samples were positive for at least one *Lactobacillus* with occasional doubt signal for *S. salivarius* (C_T at 37); signal for *E. faecalis* was occasionally detected (C_T between 24 and 27) but always in combination with a detection of *Lactobacillus* at C_T between 15 and 18. *Lactobacilli* have been clearly detected also on sanitary towels (C_T between 22 and 25). The two fecal samples did not show a clear pattern, presenting in one case amplification signals for several bacteria with C_T between 28 and 31 with a little predominance of *E. faecalis*, while in the second case it was possible to detect the only a signal for *S. salivarius* at C_T 28.

4. Discussion

The identification of vaginal fluids is a prominent need in forensic science. At the moment no validated protocols are commonly accepted for this kind of investigation and comparative interlaboratory evaluations can strongly support technical development. A key element of the described collaboration was the determination of the efficacy and reliability of the ForFLUID kit in identifying vaginal samples. Four homogeneous vaginal samples were processed by eight laboratories and very consistent results were obtained. Regarding the amplification of *Lactobacilli*, all laboratories obtained strong signals with only two weak amplification results (samples v1 and v2 processed in laboratoy E). In all situations the *Lactobacillus* signal was the predominant one, irrespective of the applied DNA extraction kit, the type of Taq polymerase and the thermocycler model. The low accuracy level of sample v3 is mainly due to the presence of very low signal of *E. faecalis*, a common contaminant of female genitalia: anyway the sample is recognized as vaginal fluid with high probability by 7 laboratories.

In order to better clarify the specificity of the assay self-provided vaginal and non-vaginal samples have been tested by the different

Table 2
Identification of mfDNA from the homogeneous set of swabs (v1, v2, v3, v4, v11) performed by the eight participant laboratories. a) Bacterial species amplified, with indication of DNA extraction protocols and PCR instruments. P: Promega DNA IQ Casework Pro Kit. Q: QIAamp DNA mini kit. S: GenElute Bacterial Genomic DNA Kit. 7000: Applied Biosystems 7000. 7500: Applied Biosystems 7500. 7900: Applied Biosystems 7900 HT Fast. RG: Rotor-Gene 6000. Lg: *L. gasseri*. Lc: *L. crispatus*. Sa: *S. aureus*. Ss: *S. salivarius*. Ef: *E. faecalis*. "weak": $30 < C_T < 35$. "?" (doubt): $C_T > 35$. b) Interpretation according to amplification data.

2a

	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H
Extraction	RG	7500	7500	7900	7500	7500	7000	7000
PCR	P	S	S	S	Q	S	S	S
v1	Lg	Lg	Lg	Lg	Lg (weak)	Lg	Lg	Lg
v2	Lg	Lg; Ef (weak)	Lg	Lg	Lg (weak)	Lg; Ef (?)	Lg	Lg
v3	Lg; Sa (?)	Lg; Ef (?)	Lg; Ss (?)	Lg; Ef (?)	Lg; Ef (?)	Lg; Ef (weak); Ss (?)	Lg; Ef (?)	Lg; Ef (?)
v4	Lg Lc; Ss (?) Ef (?)	Lc; Lg (weak)	Lg Lc; Ef (?)	Lc	Lg Lc	Lg Lc; Ss (?) Ef (?)	Lg Lc; Ef (?)	Lg Lc
v11	nd	nd	nd	nd	nd	nd	nd	nd

2b

	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Accuracy[a]	Precision
v1	Identified	Identified	Identified	Identified	Highly probable	Identified	Identified	Identified	88%	88%
v2	Identified	Probable	Identified	Identified	Highly probable	Highly probable	Identified	Identified	63%	63%
v3	Highly probable	Identified[b]	Highly probable	Highly probable	Highly probable	Probable	Highly probable	Highly probable	13%	75%
v4	Highly probable	Identified	Identified[c]	Identified	Identified	Highly probable	Identified	Identified	75%	75%
v11	nd	nd	nd	nd	nd	nd	nd	nd	100%	100%

[a] Considered only clear identifications.
[b] Specific considerations suggested as identified.
[c] The double presence of Lg and Lc and the presence of only doubt signal for Ef allowed clear identification as vaginal fluid.

laboratories. No false positives were present, due to the fact that saliva samples were always negative for *L. gasseri* and *L. crispatus* and the two fecal samples gave unclear results. This output on fecal sample is clearly in line with previous observations. In fact, as described in Giampaoli et al.[18] *Lactobacilli* sporadically can be present in fecal samples, but with a reduced titer when compared to other intestinal species. In addition, the absence of an internal positive control, partially limit the results interpretation when the ForFLUID kit is used on fecal samples, that are strongly characterized by polymerase inhibitors. One approach to mitigate PCR inhibition from organic compounds present in fecal samples is to dilute extracted DNA, as suggested in this study, where participants were asked to test samples at two concentrations: unfortunately, this dilution step can affect sensitivity in some exiguous samples.[19] It is also important to remember that the multiplex approach of the ForFLUID kit is an added value precisely for ambiguous samples: the analysis of only the presence of *Lactobacilli* could lead to a wrong identification of some fecal samples, generating a false positive result. The principle of microbial signature analysis can minimize this kind of mistake, strongly reducing the false positive rate of the test. This was also confirmed with the results for the samples provided by the participating laboratories.

From our knowledge this is the first interlaboratory evaluation of an assay based on mfDNA identification applied to vaginal fluids for forensic biology purposes. The participation of a relatively high number of laboratories from Legal Medicine and Forensic Biology institutes of different countries is a strong and affordable point of the evaluation. While the ForFLUID kit still shows some limits, mainly due to the absence of an internal positive control, it is an interesting molecular tool that could be easily adopted by forensic geneticists and allow deeper analysis of caseworks.

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None.

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